The unfolded protein response in glioblastoma stem cells: towards new targets for therapy

Peñaranda Fajardo, Natalia

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Chapter 6

Summary, Discussion and Future Perspectives

Summary

Glioblastoma (GBM) is the most common brain tumor in adults and has highly aggressive properties resulting in a very poor prognosis with a survival rate lower than 10% after 2 years for *IDH-wt* GBM [1]. Resistance to current therapies and highly invasive tumor growth in the brain limits extensive surgical resection of the tumor making GBM difficult to treat [2]. GBM stem cells (GSCs), a particular malignant subpopulation of tumor cells found in GBM, are deemed responsible for these aggressive properties of GBM [3]. GSCs can also generate differentiated off-spring that are generally considered less aggressive and more sensitive for treatments [4]. Our current knowledge of the mechanisms that support GSC maintenance and GBM progression are incomplete. There is a great need for the identification of better targets for the development of more effective therapy, which according to the cancer stem cell hypothesis should eliminate GSCs in addition to bulk tumor cells. Targeting mechanisms that sustain GSCs is anticipated to weaken their self-renewal capacity, growth and aggressive properties and may provide directions for improving therapy for patients.

The unfolded protein response (UPR) is an adaptive quality control mechanism that maintains protein homeostasis in the ER in cells. ER stress such as caused by hypoxia, nutrient/ glucose shortage as well as therapeutic agents can impair protein homeostasis resulting in activation of the UPR. Three ER membrane localized stress sensors, IRE1, PERK and ATF6, coordinate the UPR ultimately resulting in cell survival or cell death [5-7]. In cancer cells, including GBM, the UPR is chronically activated as a result of persistent ER stress due to a harsh tumor microenvironment and a high demand on protein synthesis that supports tumor growth. Furthermore, roles for the UPR in oncogenesis, metastatic potential, genomic stability, stem cell status, angiogenesis, immunogenic tolerance and metabolic status of tumor cells have been established, which has greatly encouraged research to the potential therapeutic exploration of the UPR [8-10]. In GBM the importance of the UPR has been demonstrated in tumor progression and therapy resistance [11]. However, studies on the importance of the UPR in drug sensitivity and stem cell properties of GSCs are largely absent.

The main aim of the work described in this thesis was to examine the importance of the UPR in maintaining GSCs, to elucidate underlying mechanisms and explore possibilities for targeted therapy.

Chapter 1 provides a general introduction on GBM, GSCs and current therapeutic challenges in this deadly tumor. The UPR is introduced as a possible target for therapy in cancer, specifically focusing on GBM, and the aim and outline of the thesis are described.

In **chapter 2** we reviewed the current knowledge of UPR functioning and therapeutic opportunities in GBM. In several cancer types UPR signaling has been found to regulate tumor growth, therapy sensitivity and tumor progression. From this literature overview it is clear that the role of the UPR in gliomagenesis and GSC maintenance is largely unknown. This thesis attempts to fill in this knowledge gap, in particular focusing on the impact of ER stress and the UPR on GSC maintenance.

In **chapter 3**, first we examined the possible prognostic value of several UPR markers in GBM patient samples on a tissue microarray (GBM TMA). The expression of GRP78/BIP, XBP1 and ATF4 was determined by immunohistochemistry (IHC) and high ATF4 levels was found to correlate with worse prognosis, suggesting importance of the PERK pathway in this context. We continued by studying ER stress sensitivity in our GSC-enriched GBM neurosphere models and their serum-differentiated counterparts, showing particular high sensitivity of GSCs to ER stress. The PERK-eIF2α-ATF4 canonical pathway was identified as a main mediator of ER stress cytotoxicity. Interestingly, ER stress potently inhibited self-renewal potential of GSCs accompanied by downregulation of the stem cell transcription factor SOX2 at the protein level. This was mediated by PERK but did not require eIF2α or ATF4. In addition, genetic ablation of PERK in GBM neurospheres resulted in impaired differentiation likely indicating that differentiation-induced disturbances in protein homeostasis also require PERK signaling for restoring homeostasis. Taken together, a novel noncanonical mechanism for PERK was identified that regulates SOX2 and stemness in GSCs. The role of PERK in GSCs appeared to be pleiotropic and involve canonical and noncanonical mechanisms. We conclude that ER stress-inducing therapies and PERK modulation may provide promising therapeutic approaches in GBM. In the appendix of chapter 3 the expression of the hypoxia marker GLUT1 was determined in the GBM TMA and correlated to the assessed UPR markers. Hypoxic regions are characteristic for GBM and oxygen shortage has been linked with UPR activation. No correlation was found in the evaluated cohort between GLUT1 expression and overall survival, however significant co-expression was found between GLUT1 and nuclear XBP1, suggesting

association between hypoxia and UPR activation. In addition, we elaborated on digital image analysis used for quantification of UPR marker expression in the GBM TMA. The advantages of this technique that improves objectivity, reproducibility and quantification of scoring is illustrated.

In **chapter 4** we explored if pharmacological PERK inhibition with GSK2606414 (GSK414) or PERK genetic knockout in the absence of acute ER stress had an impact on GSC self-renewal potential. Indeed, PERK was found to regulate this property of GSCs that was not accompanied by SOX2 downregulation suggesting a mechanism distinct from acute ER stress (or differentiation) induced inhibition of self-renewal. In order to identify possible underlying mechanisms comparative transcriptomics was performed using the GBM neurosphere models, GG16 and GSC23, and their differentiated counterparts. First, GSK414 exposure showed only a limited number of differentially expressed genes (DEGs) in both models that may play a role in GSC maintenance. GO analyses indicated downregulation of DNA repair and catabolic processes and upregulation of wound healing and vasculature-related processes upon GSK414 treatment of GG16 cells, whereas in GSC23 downregulation of ER- and UPR-related processes and upregulation of RNA translation related processes were seen. Second, DEGs were identified between GBM neurospheres and their serum-differentiated counterparts allowing identification of genes involved in GSC maintenance and differentiation. This revealed a large number of DEGs,1747 and 2482 in GG16 and GSC23, respectively. GO analyses of GG16 DEGs revealed downregulation of GTPase and RAS signal transduction, sterol/cholesterol biosynthesis and actin cytoskeleton-related processes, and upregulation of processes related to protein translation and ER protein targeting. In GSC23 differentiation resulted in downregulation of chromatin and histone modification processes, peptidyl-threonine modifications and (neural) development processes, whereas electron transport chain processes, extracellular matrix organization and cytokine and immune-related processes were upregulated. Comparing DEGs of GG16 and GSC23 showed around 25% overlapping DEGs, indicating heterogeneity in the response to serum-induced differentiation. This was also illustrated by differences in expression of known stem cell markers. For example, the stem cell markers *OLIG2* and *MYC/MYCN* were decreased in differentiated GG16 and GSC23 cells, whereas surprisingly stem cell marker *CD44* increased. GSEA of overlapping DEGs displayed downregulation of G2/M checkpoint and mitotic spindle

regulatory mechanisms, androgen and estrogen responses, TNFα, MTORC, KRAS and IL2/STAT5 signaling. Upregulated DEGs were associated with pathways related to inflammatory, interferon gamma and alpha responses, and epithelial-mesenchymal transition (EMT). Finally, overlapping DEGs in the GSK414 treatment and differentiation datasets were found only in the GG16 model (7 down- and 23 upregulated). GSEA indicated an upregulation of shared DEGs linked with apoptosis, EMT and TNF signaling. *CD44* and *TNC* were among the DEGs affected by both PERK inhibition and differentiation and may be interesting candidates for further studies. Taken together, comparative transcriptomics provided some mechanistic clues for PERK-dependent regulation of GSCs. However, the effect of PERK inhibition in absence of acute stress on transcription was modest and indicates that PERK functioning at the protein level should also be evaluated to obtain insight in the underlying mechanism.

In **chapter 5** we examined the possible contribution of autophagy in maintaining cellular homeostasis in GBM neurospheres and differentiated counterparts upon ER stress induction by thapsigargin in three of our GBM neurosphere models including GG16 and GSC23. Thapsigargin exposure resulted in time-dependent activation of autophagy markers as well as apoptotic markers. Autophagy activation apparently did not differ between neurospheres and differentiated counterparts. Thapsigargin had variable effects on the autophagic flux and co-exposure with the autophagy inhibiter BafA1 potentiated thapsigargin cytotoxicity in all three GBM models, however, reaching significance only in GG6 neurospheres. To examine possible differences in autophagy gene expression in GBM neurospheres an differentiated-counterparts transcriptomic analyses revealed lower levels of genes active in autophagosome formation in neurospheres compared to differentiated cells, which may suggest a higher dependency on autophagy for cell survival in GBM neurospheres. Taken together, this preliminary study shows that ER stress induction by thapsigargin leads to autophagy activation in all tested GBM models and a possible higher dependency of GBM neurospheres for survival compared to their differentiated counterparts. Additional work is required to substantiate these findings and in addition examine the molecular mechanisms that link ER stress to autophagy and relevance for GSC maintenance.

Discussion and Future Perspectives

PERK as a regulator of GSCs via noncanonical mechanisms

Our studies have identified the PERK branch of the UPR as an important regulator of GSCs stemness and differentiation. Interestingly, the regular (canonical) PERK-eIF2α-ATF4 pathway was not required for this effect, but PERK itself, thus discovering a novel noncanonical function of PERK. The importance of the UPR and specifically the PERK pathway has been shown before in other tumor types. Colon cancer stem cells were demonstrated to differentiate upon ER stress that was accompanied with increased sensitivity for chemotherapy [12,13]. PERK-eIF2α signaling was instrumental for ER stress-induced differentiation, different from our findings in GBM neurospheres where ER stress did not lead to an increase in expression of the differentiation markers examined. More recently, the same research group showed that overexpression of both active ATF6 and XBP1 resulted in decreased proliferation and stemness in a colorectal cancer stem cell model [12,13]. Interestingly, XBP1s-mediated suppression of stemness required cross-activation of PERK-eIF2α placing this pathway central in stemness regulation. Also in breast cancer stem cell models, recently a key role of the UPR and PERK in stem cell regulation was demonstrated. Cancer stem cells (CSC) enriched mammospheres displayed increased UPR activity compared with non-CSC and inhibition of all three UPR sensors could suppress breast cancer growth and self-renewal of the CSC population in *in vitro* and *in vivo* experiments [14,15]. On the other hand, tunicamycin and thapsigargin also reduced mammosphere formation and it was suggested that an appropriate level of UPR activation is essential for CSC maintenance. These findings in mammospheres resemble our findings in GBM neurospheres were ER stress also resulted in inhibition of stemness and, similarly, by PERK inhibition in absence of extrinsic stress (Chapter 3 and 4). Furthermore, in these mammospheres ER stress decreased OCT4, SOX2 and c-MYC levels, and SOX2 downregulation could be mainly linked to PERK and ATF6 activity. However, distinct from our findings, regulation of SOX2 occurred at the transcriptional level, involving interactions between ATF4, ATF6, the CCAAT-enhancer-binding protein delta (C/EBPδ) and SOX2 thus providing a mechanism for CSC maintenance [14,15]. Besides playing an important role in CSC maintenance the UPR and PERK also

regulate differentiation of normal stem cells. For example, PERK-eIF2α was required for differentiation of epithelial stem cells in the intestine [16]. Also proper functioning of satellite stem cells that control skeletal muscle repair required the PERK pathway, likely via p38MAPK, thus regulated homeostasis during regenerative myogenesis [17]. Taken together, our findings in GBM neurospheres support a critical role of the UPR and particularly PERK in regulating stemness in malignant tissues.

As described above, the underlying mechanism by which PERK-eIF2α-ATF4 regulates stemness has been partially elucidated thus far. Contrary to this mechanism we found an eIF2α-independent noncanonical mechanism to regulate stemness in GBM neurospheres. Noncanonical mechanisms for PERK have been reported before. Protein binding of PERK to the actin-binding protein Filamin A (FLNA) was found to be instrumental for ER-plasma membrane (PM) interactions [18,19]. This interaction was essential for maintaining cellular Ca^{2+} homeostasis that involved PERK-FLNA-mediated regulation of F-actin remodeling thereby enabling the formation of ER-PM interaction to control Ca^{2+} levels after ER storage depletion. The same research group showed earlier that PERK also plays a role in inducing ROS-dependent apoptosis by mediating ER-mitochondrial membrane juxtaposition [18,19]. PERK deficiency may thus reduce cell death activation through ROS-inducing agents. Another noncanonical role for PERK has been demonstrated in the emission of two major damage-associated molecular patterns (DAMPs), surface-exposed calreticulin and secreted ATP, which are key for triggering immunogenic cell death [20]. The precise mechanism was not determined but likely involves the known role of PERK in regulating the secretory pathway [21]. More recently, IRE1α was demonstrated to have a similar scaffold function as PERK by binding to FLNA and regulate cytoskeleton remodeling [22]. Moreover, IRE1α deficiency impaired cellular migration in various preclinical models, and affected normal brain development in mice.

It remains to be examined if the noncanonical mechanisms described above are also involved in regulating stemness in GBM neurospheres. As a strategy to identify novel PERK-dependent molecular mechanisms we performed comparative transcriptomics to identify possible PERK-regulated genes (Chapter 4). Although the effect of PERK inhibition in the absence of acute ER stress on transcription was only modest in GG16 and GSC23, a considerable heterogenic response to treatment with the PERK inhibitor GSK414 and upon serum-differentiation was seen. Regardless,

several overlapping genes could be identified that may play a role in stemness regulation. However, these candidate genes need further validation and it remains to be explored how PERK can regulate transcription independent from eIF2α. It is likely that experiments designed to identify possible novel PERK protein interactors will provide a promising strategy to unravel how PERK regulates stemness in GBM, either in presence of acute ER stress and through modulation of SOX2 protein expression or independent of SOX2 in the absence of extrinsic stressors.

Finally, the UPR is known to activate autophagy as a cell survival mechanism in tumor cells as well as maintenance of CSCs [23,24]. PERK-ATF4 signaling has been shown to mediate ER stress-induced autophagy activation and IRE1 was identified as a negative regulator [25]. Similarly, autophagy may contribute to GBM neurosphere/ GSC survival when challenged with ER stress, based on our preliminary findings described in Chapter 5. Further examination to UPR-induced autophagy, and particular the role of PERK herein, may reveal novel mechanisms of cell survival and stemness maintenance in GBM neurospheres.

PERK contradictions and therapeutic implications

Our results show an association between elevated expression of ATF4, an important downstream effector of the PERK pathway, and worse prognosis in GBM patients. Considering the protective function of the UPR against both cell intrinsic and extrinsic ER stressors including chemotherapy resistance, high ATF4 levels may reflect enhanced protective activity thus supporting GBM cell survival and tumor growth. This is in line with other studies. For example, a recent report demonstrated that PERK inhibition sensitized colon cancer cells for 5-FU in cell culture and xenograft mouse models [26,27]. On the other hand, this notion seems contradicting to our *in vitro* studies were we found that PERK mediated ER stress-induced cytotoxicity in the GBM neurosphere models. Cytotoxicity was mediated by PERK-eIF2α-ATF4 activation and was associated with CHOP accumulation and cell death activation. It should be noted that inhibition of the PERK branch only partially reduced ER stress-induced cell death, suggestive of redundancy in UPR mechanisms that activate cell death. The UPR balances cell survival and cell death decisions in cells based on the integration of

various signals related to protein quality control and maintaining proteostasis. Variable levels of intrinsic and extrinsic ER stress and cell specific variation in UPR status may lead to diverse outcomes upon ER stress-inducing agents. This is also illustrated for example by a report showing both a cell survival and cell death function of PERK in irradiated glioblastoma cells, mediated by the PERK-eIF2α-ATF4 pathway [28]. In this study combined irradiation and sustained activation of eIF2α, by inhibiting the GADD34 negative feedback loop, resulted subsequently in prolonged activation of the PERK-eIF2α-ATF4 route, CHOP induction and cell death activation. Thus, sustained UPR activation resulting in cell death/apoptosis activation in tumor cells is expected to be beneficial for prognosis. From the IHC studies shown in Chapter 3 it is difficult to distinguish a pro-death or pro survival status of the UPR. To further explore the contribution of ATF4 to GBM progression, additional markers should be tested on the GBM TMA such as active phosphorylated-PERK and/or p-eIF2α as well as the CHOP to corroborate temporal or chronic PERK activation. In addition, analyses of SOX2 expression in the TMA may further link PERK signaling with stemness regulation, although it should be noted that we also observed SOX2-independent regulation of stemness by PERK in GBM neurospheres. Furthermore, validation of the prognostic value of ATF4 or other critical players in the PERK pathway is required by extending the patient cohort and/ or evaluation in additional patients cohorts.

The dual role of the PERK pathway in cell death and survival activation and the identified new role of PERK as a regulator of stemness in absence and presence of acute stress appears to complicate the design of effective PERK targeted therapies. PERK targeted therapy provides a double-edged sword potentially resulting in both cytoprotective and cell death inducing activity depending on cell type and UPR status. Identification of biomarkers predicting PERK activity status may be helpful for patient stratification. On the other hand, a more pragmatic approach could be taken, in which patient-derived tumor cells are propagated as neurospheres or organoids allowing testing for sensitivity to PERK inhibitors alone or concomitantly with radiation or TMZ treatment. Currently, the use of available pharmacological PERK inhibitors in mice, such as GSK2606414, result to strong toxicity in the pancreas causing imbalanced glucose metabolism and diabetes, a phenotype that was earlier also reported in PERK knockout mouse [29,30]. Inhibition of eIF2α with the drug ISRIB did not demonstrate pancreatic toxicity [31]. However, the pharmacological properties of ISRIB are not

favorable for clinical use. Interestingly, the repurpose drug Trazodone, a clinically used antidepressant, was found to have similar activity as ISRIB and showed no systemic toxicity in mouse models [32]. Regardless of this, more in depth preclinical examination of PERK pathway signaling and its precise mode of action will be required to identify potential novel targets for developing drugs with less side effects, and to provide a rational for optimal treatment

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